The proteomic response of larvae of the Sydney rock oyster, *Saccostrea glomerata* to elevated $p\text{CO}_2$

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ABSTRACT

The acidification of oceans is predicted to fundamentally alter marine ecosystems. Previous studies have found that elevated CO_2 has an effect on adult calcification, fertilisation and larval development, perhaps because of the organisms' inability to regulate acid-base status, but little is known about the mechanisms that underlie such responses. This study investigated the growth response of larvae of a wild and selectively bred line of the Sydney rock oyster, *Saccostrea glomerata*, to elevated CO_2 and measured the pattern of expression of proteins. Overall exposure to elevated CO_2 caused a significant reduction in the shell length of D-veliger larvae of the wild, but not in the selectively bred line. Prior to this study, differences in growth between selectively bred and wild oysters have only been found following settlement. Proteome analysis of D-veliger larvae using two-dimensional gel electrophoresis detected a significantly greater number of protein spots in selectively bred compared to wild oyster lines. In addition, a comparison of the proteins expressed between selectively bred and wild larvae exposed to elevated CO_2 and ambient conditions showed that a number of proteins were up- or down-regulated and in some cases, switched on at elevated CO_2 in selectively bred lines, but not found in the wild lines. Identification of these differentially expressed proteins may assist to "climate proof" of important aquacultural industries.

Key words: ocean acidification, climate change, *Saccostrea glomerata*, Sydney rock oyster, selective breeding, spat, genetic differences, carbon dioxide, aquaculture, adaptation, proteomics.

Introduction

Acidification of oceans is predicted to fundamentally alter marine ecosystems. It is anticipated that by 2100, the concentration of CO_2 may double preindustrial levels and reach >750ppm, and lead to changes in seawater chemistry including a reduction in pH of 0.4 units (Calderia and Wickett 2003). This change in pH will directly affect marine organisms that construct their shells and skeletons from calcium carbonate ($CaCO_3$), and will pose significant challenges to aquaculture (Gao *et al.* 1993; Bijma *et al.* 1999; Riebesell *et al.* 2000; Leclercq *et al.* 2000; Reynaud *et al.* 2003; Kleypas *et al.* 2005; Langdon & Atkinson 2005; Gazeau *et al.* 2007; Hoegh-Guldberg *et al.* 2007; Ries *et al.* 2009). Recent studies have shown that the larval stages of estuarine and marine molluscs and echinoderms, are particularly vulnerable to elevations in CO_2 (Kurihara 2008; Kurihara *et al.*, 2008; Havenhand and Schlegel 2008; Dupont *et al.*, 2008, 2010; Parker *et al.*, 2009, 2010; 2011a; Sheppard-Brennard *et al.*, 2010) but can also be resilient (Byrne *et al.*, 2009a,b; Gutowska *et al.* 2008). Parker *et al.* (2011a) found differing sensitivities in responses of D-veliger larvae of between selectively bred and wild populations of the Sydney rock oyster, *Saccostrea glomerata*, to elevated CO_2. When selectively bred and wild populations were exposed to elevated $p\text{CO}_2$, there was a 25% (selectively bred) compared to a 64% reduction in shell growth and an increase in abnormal D- veliger larvae in the selectively bred and wild lines respectively.

We know little, however, about the underlying physiological pathways, affected during exposure to elevated $p\text{CO}_2$ and the subsequent negative consequences for vital processes such as metabolism, protein synthesis, ion regulation, growth and immune response (Cameron 1986; Reipschläger and Pörtner 1996; Pörtner *et al.* 1998; Michaelidis *et al.* 2005; Metzger *et al.* 2007; Miles *et al.* 2007; Spicer *et al.* 2007; Pörtner 2008; Melzner *et al.* 2009). In recent times studies have found that exposure of marine organisms to elevated CO_2 altered gene expression (Todgham and Hofmann 2009; Stumpp *et al.* , 2011), caused changes in protein expression (Picchietti *et al.* 2009; Sveinsdóttir *et al.* 2009) or had no effect on gene expression (Zippay and Hofmann 2010). Todgham and Hofmann (2009) found that larvae of the purple sea urchin, *Strongylocentrotus purpuratus*, experienced wide-scale reductions in gene expression in four major cellular processes including biomineralization, apoptosis, cellular stress response and metabolism when reared at CO_2-induced acidified seawater (pH 7.96 and 7.88). Stumpp et al., (2011) found an up regulation of metabolic genes

Pp 1011 - 1023. Paper in *Wildlife response to disasters*, the theme of the forum of the Royal Zoological Society of NSW in October 2009, edited by Chris Dickman, Daniel Lunney and Peter Banks. *Australian Zoologist* 2011 35 (4).

and a down regulation of calcification genes in sea urchin larvae of *S. purpuratus* exposed to elevated CO_2. In contrast, Zippay and Hofmann (2010) found no effect of reduced pH in two shell formation genes; engrailed or ap24 in the red abalone, *Haliotis rufescens*, despite seeing a reduction in thermal tolerance of the species.

Such recent emergence of genomic, and more recently transcriptomic and proteomic techniques, potentially allow us to monitor changes in regulation/ expression and determine the genomic capacity of an organism. These techniques provide information on the genome of an organism (genomics) (Dupont *et al.* 2007), the messenger RNA (mRNA) molecules or 'transcripts' that the genome produces (transcriptomics) and the proteins that the genome encodes (proteomics) (López 2007). Monitoring the expression level of genes provides vital information on the content of cells i.e. DNA or RNA (López 2007). The expression level of genes, however, does not always coincide with the abundance of proteins. Transcriptional, post-transcriptional and post-translational modifications (Jellum *et al.* 1983; Stegmann *et al.* 1992; Welch 1993; Bradley *et al.* 1994; Shepard and Bradley 2000) can all influence the concentration of proteins within a cell (Thiyagarajan and Qian 2008). For this reason, monitoring protein expression patterns of organisms may be used to complement gene expression analysis and enhance our understanding of the underlying mechanisms involved during exposure to elevated CO_2. Moreover, changes in gene and/or protein expression have been reported for various species of oysters during exposure to thermal stress (Damiens *et al.* 2004; Piano *et al.* 2004; Ueda and Boettcher 2009), salinity stress (Damiens *et al.* 2004; Butt *et al.* 2006), heavy metals (Tanguy *et al.* 2001; Piano *et al.* 2004), organic pollutants (Keppler and Ringwood 2001; Medeiros *et al.* 2008), disease and infection (Peters and Raftos 2003; Cao *et al.* 2009; Dheilly *et al.* 2009; Simonian *et al.* 2009a,b) and recently elevated CO_2 (Todgham and Hofmann 2009; Stumpp *et al.* 2011).

The aim of this study was to use proteomics to investigate why larvae of some selectively bred lines of the oyster *S. glomerata* show greater resilience compared to wild populations to elevated CO_2 (Parker *et al.*, 2011), whether such techniques are useful to identify potential markers of CO_2 sensitivity and/or resilience (Sveinsdóttir *et al.* 2009) and provide essential information on the molecular and biochemical pathways associated with CO_2-stress. It is hypothesised that acute exposure of larvae to elevated CO_2 will cause differences in the pattern of expression of proteins between selectively bred and wild larvae.

Materials and methods

Organisms and treatments

Two lines of *S. glomerata* larvae were used for this study; a wild (W) line produced from parents collected from two major oyster growing estuaries in NSW, Wallis Lake (32°10' S, 152°29' E) and Port Stephens (32°45' S, 152°10' E), and a 6th generation selectively bred (SB) fast growth line produced from a population of selectively bred parents held at Port Stephens (32°45' S, 152°10' E) NSW. Gravid *S. glomerata* from each population were selected at random from these locations and returned to the hatchery. Selectively bred oysters were naturally spawned while wild oysters were stripped spawned. In both instances gametes from a minimum of 10 males and 10 females were used to ensure genetic diversity and larvae were reared for 4 d before use to ensure that the method of spawning had no effect on development. The gametes from both oyster lines were filtered through a 45 μm (sperm) and 63 μm (eggs) nylon mesh and were transferred into separate 20 L buckets (1 μm FSW; 25 °C; 35 ppt) and allowed to fertilise. Following successful fertilisation, wild and selectively bred embryos were transferred into identical 1000 L fibreglass larval rearing tanks, filled with 1 μm FSW (25 °C; 35 ppt) at a concentration of 10–15 embryos mL^{-1} and were gently aerated. Larval feeding began with the appearance of the first D-veligers, after approximately 16 h. One ambient (375 ppm) and one elevated (1000 ppm) concentration of pCO_2 were selected for this study, based on the $CaCO_3$ saturation levels and projections by the IPCC (Houghton *et al.* 1996, 2001; Solomon *et al.* 2007) for likely ambient pCO_2 and temperature outcomes for 2100. There were three replicate samples for each pCO_2-oyster line combination.

To collect enough larvae for two-dimensional electrophoresis (2DE), the experiments were set up in 200 L fibreglass tanks. Prior to the experiment, 12 x 200 L tanks were thoroughly washed with Virkon S solution (Antec Corp), rinsed with freshwater and left to air dry for 24 h (O'Connor *et al.* 2008). The tanks were then fitted with a tap and air stone and were filled with 1 μm FSW (25 °C; 35 ppt) collected from Little Beach (152°07' E, 32°72' S), Nelson Bay, NSW. The elevated pCO_2 concentration was obtained in 6 of the tanks by a manipulation of pH by direct bubbling of CO_2 in seawater (mean TA = 2284 ± 30 (S.E.) μmol kg^{-1}; Gran 1952; Butler 1982). Once the pH was obtained, all 12 tanks were sealed with polyurethane plastic bags to minimise gas exchange and pH was monitored throughout the experiment.

Within 30 min of sealing the tanks 4 d old D-veliger larvae were added into each tank at a concentration of 2.5 larvae mL^{-1} (5×10^5 larvae per tank). The initial size of the wild and selectively bred larvae was 87.86 ± 2.17 (S.E.) μm and 88.38 ± 2.52 (S.E.) μm, respectively. Larvae were fed 175 mL of a combined algal diet twice daily consisting of 40% *C. calcitrans*, 10% *C. muelleri*, 25 % *P. lutheri* and 25 % T. *Isochrysis aff. galbana* on a dry weight basis (O'Connor *et al.* 2008) and there was a complete water change after 2 d for each replicate sample. After 4 d in the treatments the experiment was stopped and larvae were collected for 2DE analysis. Each tank was drained and larvae were retained on a 35 μm screen. A subsample of larvae was taken from each tank and preserved in 5% buffered formalin and the length of the shell of 30 larvae in each replicate sample was quantified, using a Sedgwick-Rafter slide under a compound light microscope (*Leica* 100x). The remaining larvae from each tank were then washed into 15 mL centrifuge tubes (one tube per 200 L replicate tank). The larval samples were frozen immediately in liquid nitrogen and were stored in a -80 °C freezer awaiting proteomic analysis.

Sample preparation

This study used 2DE to determine whether elevations in atmospheric CO_2 influence the protein expression pattern of D-veliger larvae of *S. glomerata*. 2DE is a highly effective proteomic technique which allows the simultaneous analysis of hundreds of proteins representative of a sample from an organism at a particular time in its lifecycle under a particular biological condition (Zhao *et al.* 2006; Thiyagarajan *et al.* 2009). The technique has successfully increased our understanding of a number of processes in marine organisms including the complex nature of immunological responses (Simonian *et al.* 2009a) and differences in protein expression between developmental stages (Thiyagarajan and Qian 2008).

Protein isolation

Larval samples were thawed and suspended in 1 μm FSW and kept on ice at 4 °C. Larvae from each replicate sample were equally divided into three 1.5 mL microcentrifuge tubes (36 tubes altogether). The larvae were allowed to settle to the bottom of the tubes and the overlying seawater was removed. The samples were then weighed to determine the mass of larvae in each tube. Following the weighing process, 100 μL of 1 μm FSW was added to each microcentrifuge tube and the samples were homogenised and vortexed for 2—3 min to break the shells and solubilise the larval proteins. The samples were then centrifuged at 2000*g* at 4 °C for 5 min to separate the shell from the solution and the supernatant layer was collected in a separate 1.5 mL microcentrifuge tube containing 300 μL of tri-reagent. The pellet containing shell material was discarded. To facilitate the removal of RNA from the sample 30 μL of bromochloropropane (BCP) was added to each supernatant—tri-reagent solution which was then shaken vigorously for 15 sec and stored at room temperature (20 °C) for 15 min. After centrifugation at 12000*g* at 4 °C for 15 min, the supernatant (clear) layer consisting of RNA was removed and discarded and the bottom (pink) layer was retained. To facilitate the extraction of DNA, 90 μL of 100% ethanol was added to each sample. The samples were mixed by inversion, stored at room temperature (20 °C) for 3 min and centrifuged at 2000*g* at 4 °C for 5 min. The supernatant containing the protein was retained and the DNA pellet was discarded.

Protein precipitation, washing and quantification

Ice cold acetone (3 x the volume of the protein supernatant) was added to precipitate the proteins. The samples were inverted for 10—15 sec to mix the solution and were stored at room temperature (20 °C) for 10 min. Following centrifugation at 12000*g* at 4 °C for 10 min the supernatant was removed and the protein pellet was collected. The pellet was washed by resuspending in 1 mL of guanidine hydrochloride solution (0.3M guanidine hydrochloride in 95% ethanol + 2.5% glycerol v/v), storing the samples at room temperature (20 °C) for 10 min and centrifuging at 8000*g* at 4 °C for 5 min. The supernatant was removed and discarded and the washing process was repeated twice more. For the final 10 min wash (fourth), the pellet was resuspended in 1 mL of ethanol/ glycerol solution (95% ethanol + 2.5% glycerol v/v). After centrifugation at 8000*g* at 4 °C for 5 min, the supernatant was removed and discarded and the microcentrifuge tubes were inverted to allow the pellets to air dry for 10 min. Each pellet was resuspended in 50 μL of rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 40 mM dithiothreitol (DTT), and 1% bromophenol blue). The concentration of protein in each sample was quantified using the BioRad (Australia) quantification kit.

2DE

To prepare for the first dimension of electrophoresis, 200 μg of protein from each replicate sample (pooled from the three microcentrifuge tubes used for each replicate and made up to 125 μL with rehydration buffer, as above, with the addition of 2% Bio-Lyte 3/10 ampholyte) was loaded into a rehydration tray using a pipette. 7 cm IPG strips (BioRad), pH 3—10 (linear) were placed gel side down on the solution and left for 30 min before being covered with 1 mL of DryStrip Cover Fluid to prevent evaporation during overnight rehydration of the strips. First dimension electrophoresis was carried out by placing the IPG strips on an Ettan IPGphor II isoelectric focussing (IEF) cell (GE Healthcare) and focussing was carried out using the following voltage gradient: 100 V for 2 h, 250 V for 20 min, 5000 V for 2 h (gradient/stepped voltage), 5000 V for 2 h to give a total voltage hours of 15000-17000. At the completion of IEF, the IPG strips were reduced for 15 minutes (6 M urea, 2% sodium dodecyl sulfate (SDS), 0.05 M Tris-HCl (pH 8.8), 50% glycerol, and 2% w/v 1,4-DTT) and then alkylated for 15 min (6 M urea, 2% SDS, 0.05 M Tris-HCl (pH 8.8), 50% glycerol, and 2.5% iodacetamide (IAA)).

To prepare for the second dimension, 7 cm 12% SDS-PAGE gels were cast. The running gel (40% Acrylamide/ bis solution, H_2O (milliQ), 1.5 M Tris-HCl (pH 8.8), 10% SDS, ammonium persulfate (APS), Temed) was cast so that it was filled to within 1—2 cm from the top of the glass plates and was left to polymerise for 60 min. The stacking gel (40% acrylamide/ bis solution, H_2O (milliQ), 0.5 M Tris-HCl (pH 6.8), 10% SDS, APS, Temed) was then cast over the top of the running gel using appropriate combs for creating wells. After 30 min, following polymerisation of the stacking gel, the gels were placed in the PROTEAN mini cell system (Bio Rad), the IPG strips were loaded on top of the gels and sealed with 0.5% w/v agarose (Thiyagarajan *et al.* 2009). 7 μL pre-stained SDS-PAGE standard protein (Bio-Rad) marker was added to each gel in the appropriate well before adding running buffer to the system. The gels were run at 20 °C at 60 to 110 mA until bromophenol blue reached the bottom of the gel. Gels were stained overnight with Silver/blue stain and excess/ background stain removed over a period of approximately 5 h with milliQ water.

Gel analysis

Gels were scanned at an optical resolution of 300 dpi using a Canon flat bed scanner (LiDE 60) and were analysed manually using Adobe Photoshop C4 (histogram tool). Differences in protein expression between pCO_2 treatments (375 ppm and 1000 ppm) and oyster types (wild and selectively bred), including total number of proteins, proteins switched 'on' or 'off' and proteins 'up' or 'down' regulated, were compared initially by visual inspection. Each protein spot was assigned a spot number based on their location on the gel and spots which differed in expression between treatments across all three replicates were identified for further analysis. In order to quantitatively compare between gels, the gels were normalised by calculating the mean spot density (number of pixels/ average brightness) of 12 randomly selected spots common to each gel which did not differ in expression. These values were calculated for each gel and were used as conversion factors. Following normalisation, the mean spot density of each protein spot suspected to differ between treatments was calculated from three replicate gels at each treatment. Due to problems in sample preparation, there were only two replicate gels for the selectively bred oyster larvae at 1000 ppm. To overcome this and enable a fully orthogonal two-way ANOVA, values for the missing replicate were obtained using a method for estimating missing observations, which minimises the interaction effect in the analysis resulting when such estimates are used in place of missing data, as described by Bennett and Franklin (1954) in Winer (1991).

Data analysis

To determine any significant differences in the shell length and protein expression pattern between oyster lines of *S. glomerata* reared at ambient and elevated pCO_2, the shell length of each line, mean number of protein spots, proteins switched 'on' or 'off' and proteins which were 'up' or 'down' regulated were analysed using a two-way ANOVA using GMAV5 (Underwood 1997), where oyster line and pCO_2 were fixed and orthogonal factors. Cochran's test was used to determine any heterogeneity of variances and data were transformed if significant. In instances where transformation did not prevent heterogeneity, interpretation was conservative because of the increased likelihood of Type I error (Underwood 1997). An SNK test was used to detect differences amongst means (Sokal and Rohlf 1995). To enable a fully orthogonal two-way ANOVA, values for the missing replicate gel were estimated (Bennett and Franklin 1954; Winer 1991).

Results

The effect of elevated pCO_2 on the shell length of D-veliger larvae

After 4 d in the treatments, there was a significant reduction in the shell length of wild D-veliger larvae reared at elevated pCO_2 (1000 ppm) compared to larvae reared at ambient pCO_2 (375 ppm). There was no significant reduction in the growth of selectively bred D-veliger larvae reared at elevated (1000 ppm) and ambient pCO_2 (375 ppm) as shown by the significant interaction between oyster line and pCO_2 concentration (Figure 1, Table 1; SNK: wild: 375 < 1000 ppm, selectively bred: 375 = 1000 ppm). Selectively bred D-veliger larvae were greater in size compared to wild D-veliger larvae when reared at elevated pCO_2, but not at ambient pCO_2 where shell length of larvae was equal across both lines (Figure 1, Table 1; Line x pCO_2 interaction SNK: 375 ppm: wild = selectively bred, 1000 ppm: wild < selectively bred).

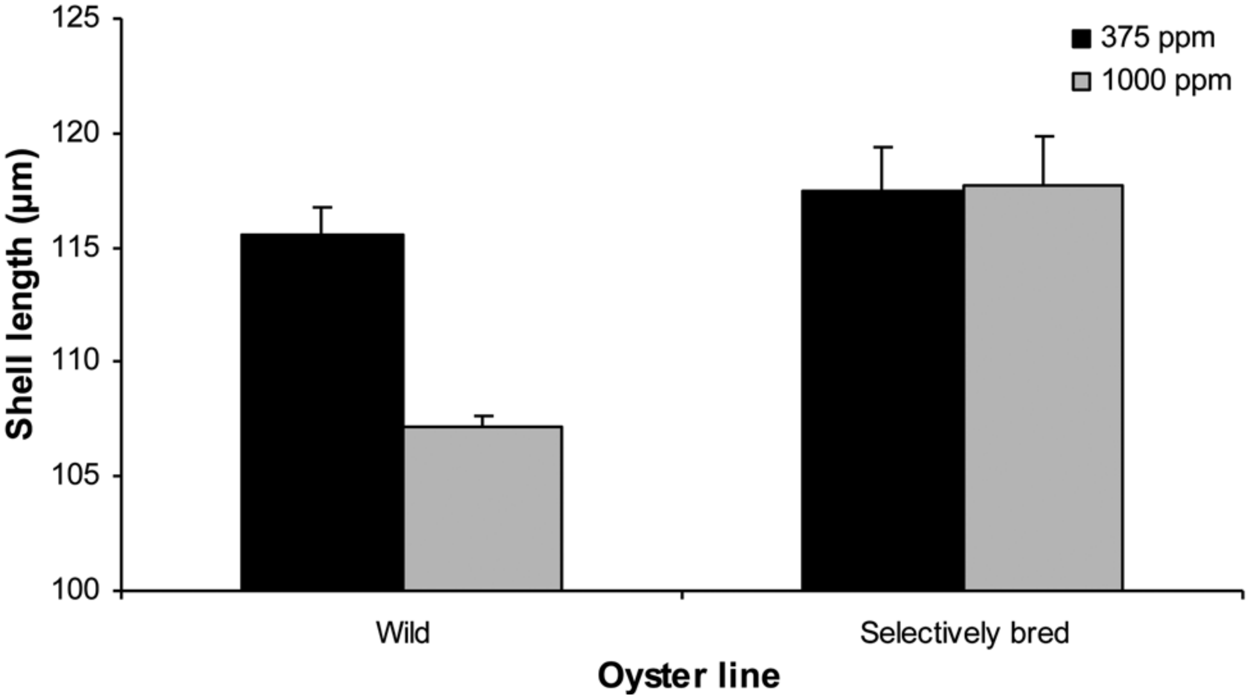

Figure 1. The mean shell growth in D-veliger larvae of *Saccostrea glomerata* from wild and selectively bred fast growth lines after 4 d at ambient (375 ppm) and elevated (1000 ppm) $_pCO2$; 25 °C, 35 ppt, n = 3 (10/01/09 to 14/01/09).

Table 1. Analysis of the mean shell growth in D-veliger larvae of Saccostrea glomerata from wild and selectively bred fast growth lines after 4 d at ambient (375 ppm) and elevated (1000 ppm) $_p$CO2; 25 °C, n = 3 (10/01/09 to 14/01/09). This was a two-way analysis with oyster line being fixed and $_p$CO2 being fixed and orthogonal. Significance level indicated by asterisks, ns = not significant, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. C = 0.49 ns.

Source of Variation	df	MS	F		SNK
Line	1	116.61	17.22	**	375 ppm: W = SB
CO$_2$	1	47.35	6.99	*	1000 ppm: W < SB
Line x CO$_2$	1	55.58	8.21	*	W: 375 < 1000 ppm
RES	8	6.77			SB: 375 = 1000 ppm
TOTAL	11				

The effect of elevated pCO$_2$ on the protein expression of D-veliger larvae

The total number of larval proteins detected differed significantly between oyster lines, but not between elevated and ambient *p*CO$_2$ levels (Figure 2, Table 2). Overall, there were 295 and 298 proteins detected in the wild and selectively bred lines, respectively at 375 ppm and 297 and 299 proteins detected in the wild and selectively bred lines, respectively at 1000 ppm (Figure 2). The proteins ranged in molecular weight from 29 to 199 kDa and isoelectric point (*p*I) from 3.5–9 (Figure 3).

There was a significantly greater concentration of five proteins at elevated CO$_2$ (1000 ppm) compared to the ambient control (375 ppm) (47, 145, 267, 312, 313; Figure 4, Table 3). For six other proteins (220, 224, 237, 268, 278, 282), the concentration of protein was dependent on elevated CO$_2$ x line (Figure 4, Table 3). Of these six proteins all were up-regulated in wild D-veliger larvae, but not in selectively bred D-veliger larvae at elevated CO$_2$. (Figure 4, Table 3). Similarly, there was significantly lower concentration of two proteins (112, 118) at elevated CO$_2$ (1000 ppm) compared to the ambient control (375 ppm; Figure 5, Table 4). For four other proteins (57, 106, 117, 196) the

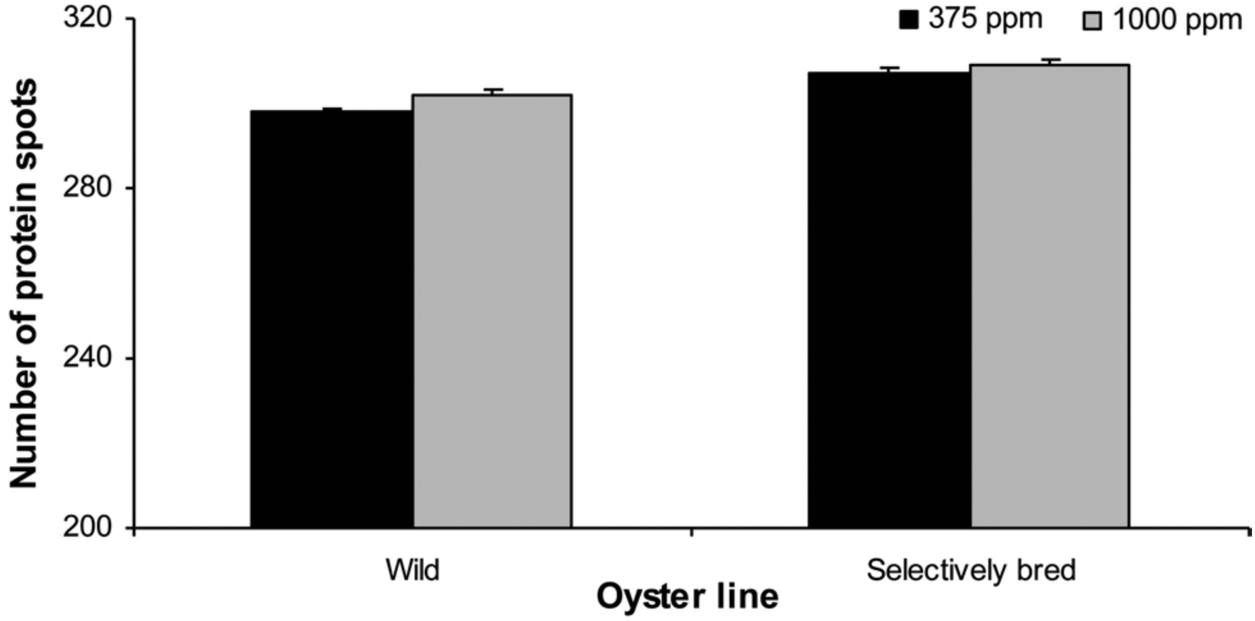

Figure 2. The mean number of proteins expressed in D-veliger larvae of *Saccostrea glomerata* from wild and selectively bred fast growth lines reared at ambient (375 ppm) and elevated (1000 ppm) $_p$CO2 for 4 d; 25 °C, 35 ppt, n = 3 (2009).

Table 2. Analysis of the mean number of proteins in D-veliger larvae of from wild and selectively bred fast growth lines after 4 d at ambient (375 ppm) and elevated (1000 ppm) $_p$CO$_2$; 25 °C, n = 3 (2009). This was a two-way analysis with oyster line bring fixed and $_p$CO$_2$ being fixed and orthogonal. Significance level indicated by asterisks, ns = not significant, * < 0.05; ** < 0.01; *** < 0.001. C = 0.34 ns.

Source of Variation	df	MS	F		SNK
Line	1	17.49	5.79	*	W < SB
CO$_2$	1	6.00	1.99	ns	
Line x CO$_2$	1	1.03	0.34	ns	
RES	8	3.02			
TOTAL	11				

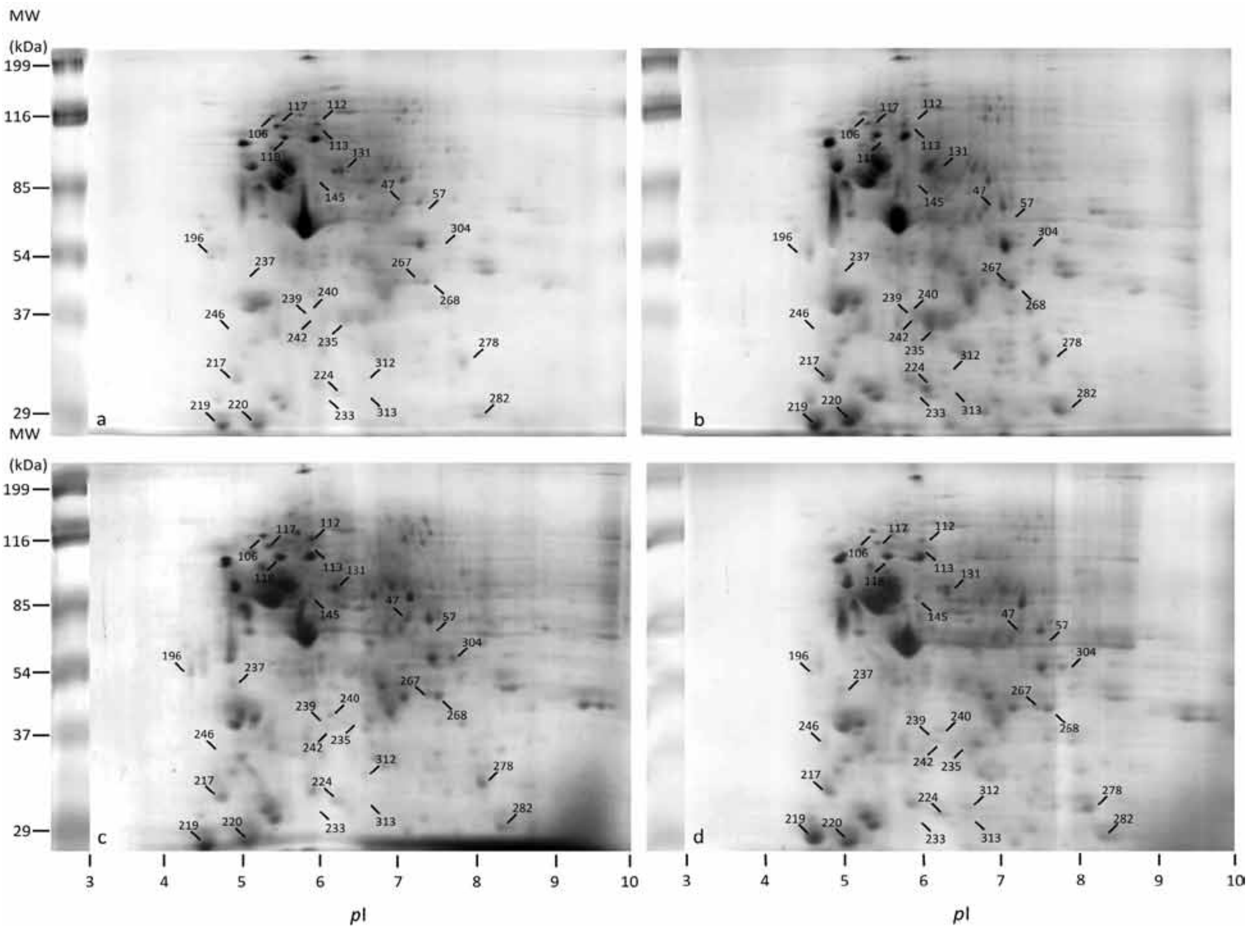

Figure 3. 2DE gels of proteins from D-veliger larvae of *Saccostrea glomerata* reared at ambient (a and c) (375 ppm) and elevated (b and d) (1000 ppm) $_p$CO$_2$ for 4 d. Gels (a) and (b) are of wild oysters and (c) and (d) are of selectively bred fast growth oysters (2009). Gels are stained with Silver/blue. Pointers represent proteins which differed in expression between pCO2¬ treatments, oyster line or both.

concentration of protein was dependent on elevated CO$_2$ and line. Of these proteins three (57, 117, 196) were down-regulated in wild D-veliger larvae, but not in selectively bred D-veliger larvae at elevated CO$_2$ (Figure 5, Table 4). The remaining protein (106) was down-regulated in both lines at elevated CO$_2$ but was higher in concentration in the wild D-veliger larvae compared to the selectively bred D-veliger larvae at ambient CO$_2$ (Figure 5, Table 4). Furthermore, proteins 113 (MS = 25.68, F = 280.91, = ***, SNK: W 1000 ppm > all other treatments) and 233 (MS = 9.06, F = 13.70, = **, SNK: W 1000 ppm > all other treatments) were switched on at 1000 ppm in wild D-veliger larvae and protein 246 (MS = 18.85, F = 15.01, = **, SNK: SB 1000 ppm > all other treatments) was switched on at 1000 ppm in selectively bred D-veliger larvae (Figure 3).

There were also two proteins which were in greater concentration in the wild D-veliger larvae compared to the selectively bred larvae (131, 217) and one protein which was greater in concentration in the selectively bred larvae compared to the wild larvae (219) (Figures 6—7, Tables 5—6). Finally, there was one protein that was detected in the wild larvae, but not the selectively bred larvae (235: MS = 830.65, F = 190.37, = ***) (Figure 3) and four proteins detected in the selectively bred larvae, but not the wild larvae (239: MS = 25.13, F = 57.84, = ***; 240: MS = 45.58, F = 52.78, = ***; 242: MS = 16.53, F = 52.94, = ***; 304: MS = 48.65, F = 16.58, = **) (Figure 3).

Discussion

This study has shown that acute exposure to predicted near-future elevations in atmospheric CO$_2$ has the potential to significantly affect the shell size and protein expression in larvae. Exposure to elevated CO$_2$ caused a significant reduction in the shell length of D-veliger larvae of wild, but not selectively bred fast growth oysters. A similar result was found in a study by Parker *et al.*, (2011) where the shell growth of newly metamorphosed spat from selectively bred fast growth lines of from the Georges River NSW was greater than the wild population reared at elevated CO$_2$. Prior to this study, differences in growth between selectively bred and wild oysters have only been found from the spat stage onwards (pers. comm. WA O'Connor). In this study, the growth rate of D-veliger larvae was similar between the wild and selectively bred lines at ambient CO$_2$, but at elevated CO$_2$ the growth rate of D-veliger larvae was significantly greater for the selectively bred than wild larvae. Such a finding is supported in a number of studies which have found a delay in development or less development in larvae of marine

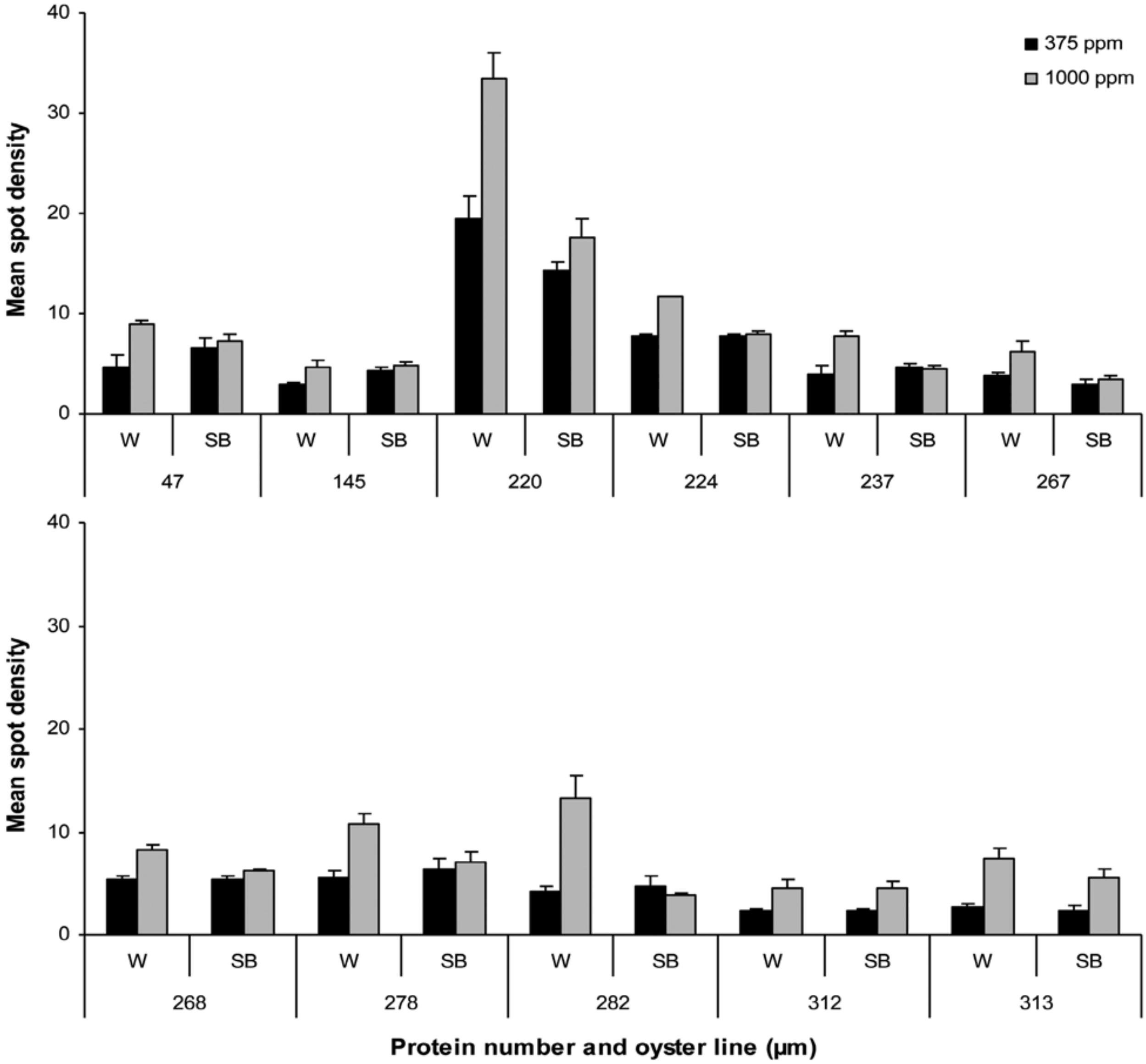

Figure 4. Proteins that were up-regulated in D-veliger larvae of from wild type and/or selectively bred fast growth lines after exposure to elevated $_{p}CO_2$ (1000 ppm) for 4 d; 25 °C, n = 3 (2009).

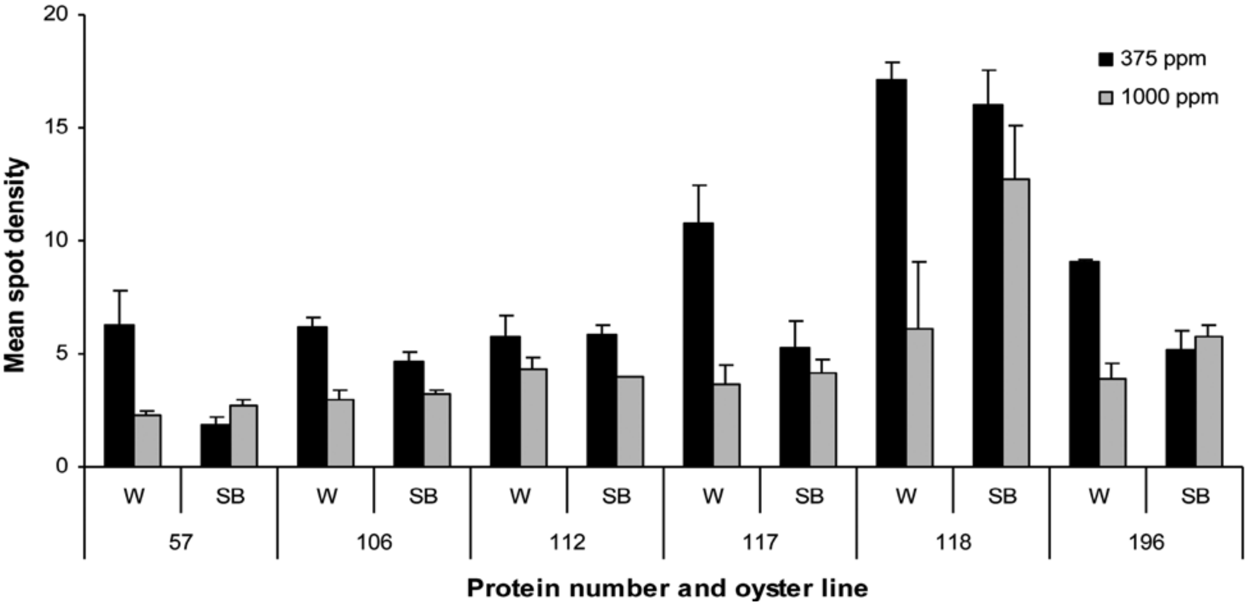

Figure 5. Proteins that were down-regulated in D-veliger larvae of from wild type and/or selectively bred fast growth lines after exposure to elevated $_{p}CO_2$ (1000 ppm) for 4 d; 25 °C, n = 3 (2009).

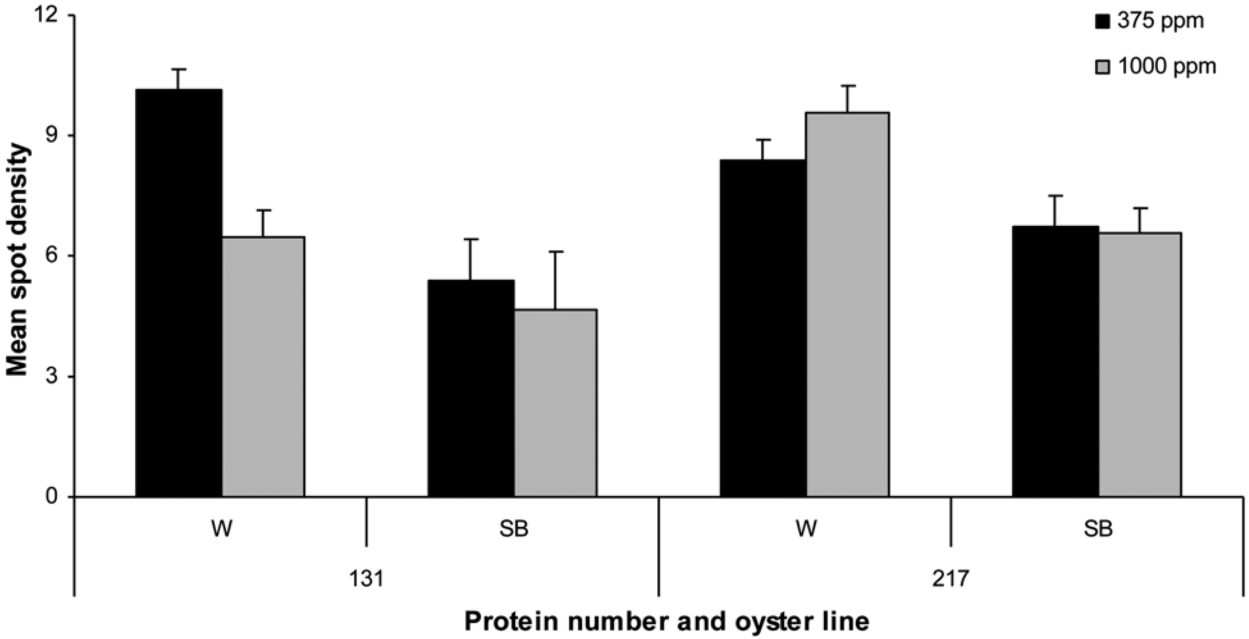

Figure 6. Proteins that were up-regulated in D-veliger larvae from wild compared to selectively bred fast growth after 4 d at ambient (375 ppm) and elevated $_{p}CO_2$ (1000 ppm); 25 °C, n = 3 (2009).

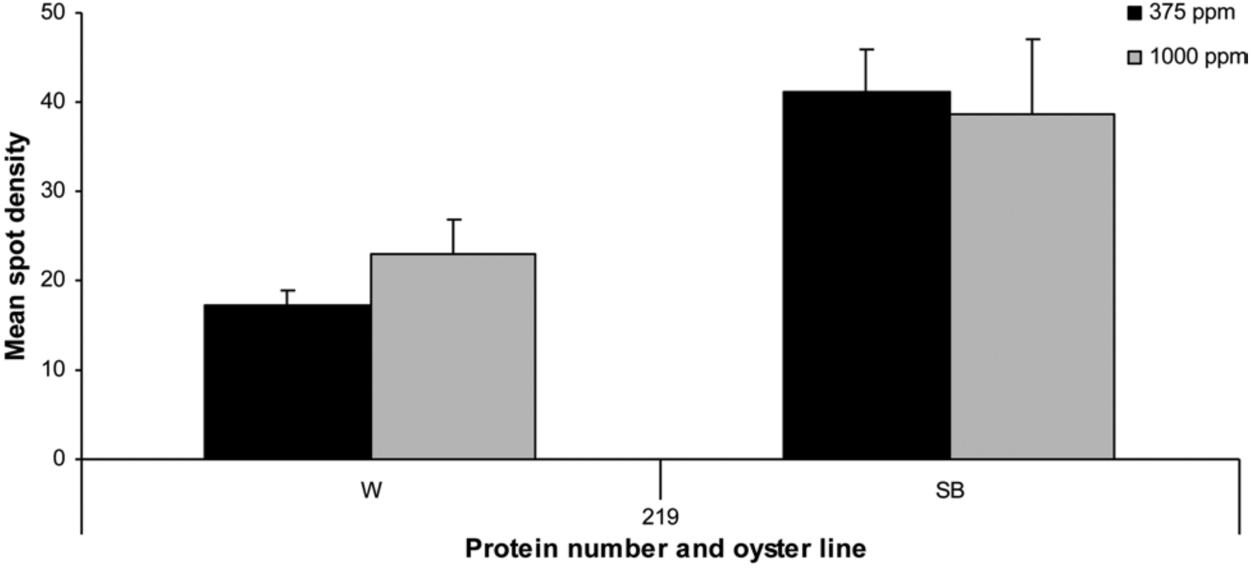

Figure 7. Protein that was up-regulated in D-veliger larvae from selectively bred fast growth line compared to wild after 4 d at ambient (375 ppm) and elevated $_{p}CO_2$ (1000 ppm); 25 °C, n = 3 (2009).

organisms exposed to elevate CO_2 including other oysters (Kurihara 2007; Parker *et al.* 2009; 2010) mussels (Kurihara 2008) the gastropods (Ellis 2009). Abnormalities in shell formation have also been found such as convex hinge and mantle protrusion in the oysters, *Crassostrea gigas* and *S. glomerata* (Kurihara *et al.* 2007; Parker *et al.*2009; 2010) the mussels [Kurihara *et al.* 2008] the gastropod, *Littorina obtusata* and in the Mediterranean pteropod, *Cavolinia inflexa* (Comeau *et al.* 2010) where shells were absent after 13 days due to dissolution, yet larvae displayed "normal" swimming action. In fewer instances there have been positive instead of negative impacts of elevated CO_2 such as in the juvenile European cuttlefish, *Sepia officinalis*, which accreted significantly more $CaCO_3$ into cuttlebones (Gutowska 2008) when raised at elevated CO_2. Differences in calcification rate between source populations of spp have also been found, signifying possible genotypic variation (Waldbusser *et al.* 2010).

Although this study found no significant difference between the total number of protein spots from D-veliger larvae reared in ambient and elevated CO_2, detected using Silver/blue stain, there was a significant difference in the number of protein spots between lines. Overall there was an average of 296 and 299 proteins detected in the wild and selectively bred lines, respectively. The current lack of proteomic studies on marine larvae in the literature makes a direct comparison of the results difficult (Thiyagarajan *et al.* 2009). A recent study by Simonian *et al.*(2009a) on a population of *S. glomerata* adults of held on the Georges River found 122 protein spots in wild adult oysters compared to 136 in selectively bred adult oysters. This study detected almost double the number of protein spots in larvae compared to the adults used in the Simonian *et al.* (2009a) study. In the only other study, to date, to report on the protein expression of bivalve larvae 350 protein spots were detected in D-veliger larvae of the

Table 3. Analysis of the proteins that were up-regulated in D-veliger larvae of from wild and/or selectively bred fast growth lines after exposure to elevated CO2 (1000 ppm) for 4 d; 25 °C, n = 3 (2009). This was a two-way analysis with oyster line being fixed and $_pCO_2$ being fixed and orthogonal. Significance level indicated by asterisks, ns = not significant, * < 0.05; ** < 0.01; *** < 0.001.

Protein		47 (C = 0.49 ns)			145 (C = 0.64 ns)			220 (C = 0.42 ns)			224 (C = 0.53 ns)			237 (C = 0.43 ns)		
Source of Variation	df	MS	F		MS	F		MS	F		MS	F		MS	F	
Line	1	0.01	0.00	ns	1.83	3.54	ns	331.43	28.08	***	10.66	87.26	***	5.67	5.73	*
CO_2	1	18.87	9.41	*	3.94	7.59	*	222.37	18.84	**	13.11	107.36	***	9.35	9.44	*
Line x CO_2	1	9.55	4.76	ns	1.27	2.45	ns	84.59	7.17	*	10.20	83.53	***	11.40	11.51	**
RES	8	2.01			0.52			11.80			0.12			0.99		
TOTAL	11															
SNK		375 < 1000 ppm			375 < 1000 ppm			375 ppm: W = SB 1000 ppm: W > SB W: 375 < 1000 ppm SB: 375 = 1000 ppm			375 ppm: W = SB 1000 ppm: W > SB W: 375 < 1000 ppm SB: 375 = 1000 ppm			375 ppm: W = SB 1000 ppm: W > SB W: 375 < 1000 ppm SB: 375 = 1000 ppm		

Table 3 Continued

Protein		267 (C = 0.54 ns)			268 (C = 0.47 ns)			278 (C = 0.34 ns)			282 (C = 0.82 < 0.05)			312 (C = 0.55 ns)			313 (C = 0.57 ns)		
Source of Variation	df	MS	F		MS	F		MS	F		MS	F		MS	F		MS	F	
Line	1	10.05	9.73	*	2.85	7.23	*	6.54	2.54	ns	56.65	12.15	**	0.002	0.00	ns	3.32	2.10	ns
CO_2	1	6.67	6.46	*	10.17	25.84	***	25.64	9.95	*	50.41	10.81	*	15.04	16.46	**	44.89	28.44	***
Line x CO_2	1	3.41	3.31	ns	2.91	7.41	*	15.08	5.85	*	74.74	16.02	**	0.01	0.01	ns	1.60	1.01	ns
RES	8	1.03			0.39			2.58			4.66			0.91			1.58		
TOTAL	11																		
SNK		375 < 1000 ppm W > SB			375 ppm: W = SB 1000 ppm: W > SB W: 375 < 1000 ppm SB: 375 = 1000 ppm			375 ppm: W = SB 1000 ppm: W > SB W: 375 < 1000 ppm SB: 375 = 1000 ppm			375 ppm: W = SB 1000 ppm: W > SB W: 375 < 1000 ppm SB: 375 = 1000 ppm			375 < 1000 ppm			375 < 1000 ppm		

Table 4. Analysis of the proteins that were down-regulated in D-veliger larvae of from wild and/or selectively bred fast growth lines after exposure to elevated CO2 (1000 ppm) for 4 d; 25 °C, n = 3 (2009). This was a two-way analysis with oyster line being fixed and $_pCO_2$ being fixed and orthogonal. Significance level indicated by asterisks, ns = not significant, * < 0.05; ** < 0.01; *** < 0.001.

Protein		57 (C = 0.92 < 0.01)			106 (C = 0.32 ns)			112 (C = 0.69 ns)			117 (C = 0.51 ns)			118 (C = 0.50 ns)			196 (C = 0.48 ns)		
Source of Variation	df	MS	F		MS	F		MS	F		MS	F		MS	F		MS	F	
Line	1	11.90	5.90	*	1.19	3.06	ns	0.06	0.06	ns	19.15	4.68	ns	22.25	1.75	ns	3.20	2.99	ns
CO_2	1	7.30	3.62	ns	16.39	42.24	***	8.48	8.61	*	50.07	12.23	**	151.92	11.92	**	15.85	14.81	**
Line x CO_2	1	17.30	8.58	*	2.33	6.02	*	0.19	0.19	ns	26.97	6.59	*	44.79	3.52	ns	24.39	22.80	**
RES	8	2.02			0.39			0.99			4.10			12.74			1.07		
TOTAL	11																		
SNK		375 ppm: W > SB 1000 ppm: W = SB W: 375 > 1000 ppm SB: 375 = 1000 ppm			375 ppm: W > SB 1000 ppm: W = SB W: 375 > 1000 ppm SB: 375 > 1000 ppm			375 > 1000 ppm			375 ppm: W > SB 1000 ppm: W = SB W: 375 > 1000 ppm SB: 375 = 1000 ppm			375 > 1000 ppm			375 ppm: W > SB 1000 ppm: W = SB W: 375 > 1000 ppm SB: 375 = 1000 ppm		

Table 5. Analysis of the proteins that were up-regulated in of D-veliger larvae of from wild compared to selectively bred fast growth lines after 4 d at ambient (375 ppm) and elevated $_pCO_2$ (1000 ppm); 25 °C, n = 3 (2009). This was a two-way analysis with oyster line being fixed and $_pCO_2$ being fixed and orthogonal. Significance level indicated by asterisks, ns = not significant, * < 0.05; ** < 0.01; *** < 0.001.

Protein		131 (C = 0.55 ns)			217 (C = 0.35 ns)		
Source of Variation	df	MS	F		MS	F	
Line	1	32.42	10.93	*	16.31	12.28	**
CO_2	1	14.30	4.82	ns	0.79	0.59	ns
Line x CO_2	1	6.26	2.11	ns	1.26	0.95	ns
RES	8	2.97			1.33		
TOTAL	11						
SNK		W > SB			W > SB		

Table 6. Analysis of the proteins that were up-regulated in D-veliger larvae of from the selectively bred fast growth compared to wild after 4 d at ambient (375 ppm) and elevated $_pCO_2$ (1000 ppm); 25 °C, n = 3 (2009). This was a two-way analysis with oyster line being fixed and $_pCO_2$ being fixed and orthogonal. Significance level indicated by asterisks, ns = not significant, * < 0.05; ** < 0.01; *** < 0.001.

Protein 219 (C = 0.65 ns)					
Source of Variation	df	MS	F	P	SNK
Line	1	1171.14	14.33	**	W < SB
CO_2	1	8.15	0.10	ns	
Line x CO_2	1	49.56	0.61	ns	
RES	8	81.75			
TOTAL	11				

mussel, M. *galloprovincialis,* using Silver staining (López *et al.* 2005). Other studies on marine larvae have detected 400 protein spots in larvae of the barnacle, *Balanus amphitrite* (Thiyagarajan and Qian 2008) and 450 protein spots in larvae of the coral, *Fungia scutaria* (deBoer *et al.* 2007) using Silver staining; 384 protein spots in larvae of the spionid polychaete, *Pseudopolydora vexillosa* using Coomassie Brilliant Blue staining (Mok *et al.* 2009); and 325 and 300 protein spots respectively, in larvae of the Bryozoan, *Bugula neritina* and barnacle, *B. amphitrite* using Sypro Ruby staining (Thiyagarajan *et al.* 2009). The large number of proteins in larvae compared to adults may represent greater biochemical and physiological changes occurring during this dynamic stage of development (Thiyagarajan *et al.* 2009)

While elevated CO_2 had no effect on the number of proteins spots detected in larvae, there was a significant effect of elevated CO_2 on the level of expression (concentration) of some larval proteins across both oyster lines. Elevated CO_2 caused a significant change in the concentration of 17 proteins in the wild oyster larvae and 5 proteins in the selectively bred oyster larvae. A large proportion of these proteins (wild: 65%, selectively bred: 80%) were up-regulated at elevated compared to ambient CO_2. This suggests that oyster larvae potentially undergo an up-regulation of genes associated with various pathways in an effort to compensate for the effects of elevated CO_2 (Todgham and Hofmann 2009; Stumpp, *et al.* 2011). In one of few studies to investigate the transcriptomic response (i.e. the set of all mRNA transcripts present in a cell, tissue or organism) of the sea urchin, *S. purpuratus* to elevated CO_2, Todgham and Hofmann (2009) found that 90% of the genes affected by elevated CO_2 in larvae of were down-regulated, with only 10% of genes being up-regulated at elevated CO_2. The majority of genes which were up-regulated were involved in the biological processes of development and cellular stress response (Todgham and Hofmann 2009). Similarly Stumpp *et al.* (2011), found between 10-20% up regulation of metabolic genes in ATP synthase, citrate synthase, pyruvate kinase and thiolase) and a 23-36% down regulation of calcification genes, msp 130, SM30E, SM50. Further work is needed to determine whether the up and down regulated proteins in this study are associated with similar processes.

Other studies, using proteomic analysis, have found an important immune defence response enzyme known as phenoloxidase in selectively bred populations of adult *S. glomerata* which are not present in the wild oyster population (Newton *et al.* 2004). Perhaps larvae from selectively bred lines have similar novel proteins or higher concentrations of existing proteins, which allow them to respond better to CO_2-induced acidification. In this study, there were two proteins at ambient and elevated CO_2 which were greater in concentration in the wild compared to the selectively bred oyster larvae and one protein that was greater in concentration in the selectively bred compared to the wild larvae. Furthermore, there was one protein present in the wild, but not in the selectively bred line and four proteins present in the selectively bred, but not in the wild line. The proteins which differ between the two oyster lines, may be involved in biochemical processes which create the enzyme carbonic anhydrase which catalyses the reversible hydration of CO_2, accelerating the formation of HCO_3^- and assisting in the construction of $CaCO_3$ shells (Wilbur & Anderson 1950, Medakovic & Lucu 1994; Medakovic 2000). Carbonic anhydrase has an important role in acid-base regulation (Kochevar &

Childress 1996; Fabry *et al.* 2008). Todgham and Hofmann (2009) found that the levels of expression of five mRNA transcripts that encode for carbonic anhydrase were affected in larvae of the sea urchin, *S. purpuratus* exposed to elevated CO_2. In contrast, Stumpp, *et al.* (2011) found no impact on the gene coding for carbonic anhydrase related protein, CA10 in response to elevated CO_2 when normalised for body length. Given the faster growth rates of the selectively bred oysters of *S. glomerata*, it is likely there is a greater concentration of carbonic anhydrase in these oysters compared to the wild population.

Unfortunately even though it is likely that some of these proteins here are stress proteins which are highly conserved across phyla, it was beyond the scope of this current study to identify the differentially expressed proteins detected because the complete sequence data for genes and proteins of are unavailable (Simonian *et al.* 2009a). Proteins 113 and 233 in the wild larvae and 246 in the selectively bred fast growth larvae are of particular interest for further analysis using mass spectrometry (MS). These proteins were switched on during exposure to elevated CO_2. When identified, knowledge of these proteins could allow a better understanding of the processes and pathways affected by elevated CO_2 and provide insight into the reasons why some species and/or populations within a species, show resilience to elevated CO_2.

This study used 2DE to detect protein changes in response to CO_2 sensitivity and resilience in larvae of the ecologically and economically important Sydney rock oyster, *S. glomerata*. Protein changes such as those found in this study represent the biochemical and physiological changes occurring in the oyster larvae in response to acute exposure to elevated CO_2. Proteomic analysis showed that specific differences in protein expression patterns occurred across both oyster lines. If we can identify these proteins, then we will have a greater understanding of how marine species are affected by elevated CO_2-stress and why some species or populations within a species show greater resilience. This information is vital not only to assist in the prediction of the future vulnerability of marine ecosystems in a high-CO_2 world, but also to help 'climate proof' important aquaculture industries, such as that of *S. glomerata*, through specific breeding/ mating of organisms with CO_2 resistant genes.

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